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NR2B downregulation in a forebrain region required for avian vocal learning is not sufficient to close the sensitive period for song learning☆,☆☆

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Abstract

The neural changes that limit the sensitive period for avian song development are unknown, but neurons in a forebrain region critical for song learning, the lMAN, exhibit experience-driven changes in NMDAR subunit expression that could regulate sensitive period closure. Specifically, NR2B levels in lMAN decrease during song acquisition, potentially reducing synaptic plasticity by decreasing NMDAR EPSC duration and/or affecting NMDAR-coupled intracellular cascades. While rearing birds in isolation extends the sensitive period and also delays the developmental changes in NR2B expression and NMDAR physiology, recent work indicates that a transition to faster NMDAR currents does not preclude further song learning. However, NR2B mRNA expression in isolates remains elevated beyond the age at which NMDAR currents shorten, leaving open the possibility that NR2B levels regulate closure of the sensitive period through effects other than those mediated by NMDAR current duration. To determine whether the experience-driven decrease in NR2B expression in lMAN closes the sensitive period, we promoted this change in gene expression either by treating isolation-reared zebra finches briefly with testosterone (T-isolates) or by allowing males limited access to conspecific song (pre-exposed isolates). We then assessed if these birds could acquire song from tutors after the normal close of the sensitive period. Despite a normal decline in NR2B expression, T-isolate and pre-exposed isolate birds learned tutor songs heard from d65-90, while normally reared birds did not. These findings suggest that the normal decline in NR2B expression with lMAN is not sufficient for sensitive period closure.

Keywords: NMDA receptor; Plasticity; Development; Androgens

1. Introduction

Sensitive periods for experience-dependent plasticity are evident in a variety of domains. While the cellular and molecular events that regulate these periods of sensitivity in the developing nervous system are unknown, much recent attention has been on changes in the composition and function of *N*-methyl-D-aspartate receptors (NMDARs) (Bear, Kleinschmidt, Gu, & Singer, 1990; Carmignoto & Vicini, 1992; Fox, Henley, & Isaac, 1999; Lu, Gonzalez, & Crair, 2001; Quinlan, Olstein, & Bear, 1999a; Quinlan, Philpot, Huganir, & Bear, 1999b; Roberts & Ramoa, 1999). This receptor is a heteromeric protein comprised of an NR1 subunit (essential for channel function), and one or more modulatory subunits (NR2A-E) (Ishii et al., 1993; Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994; Monyer et al., 1992; Sheng, Cummings, Roldan, Jan, & Jan, 1994). Several instances of developmentally regulated plasticity rely on NMDAR-mediated processes (Basham, Nordeen, & Nordeen, 1996; Goodman & Shatz, 1993; Kirkwood, Rioult, & Bear, 1996), and sensitive periods often are associated with changes in the ratio of 2B:2A NMDAR subunits (Cao, Liu, Lickey, & Gordon, 2000; Roberts & Ramoa, 1999; Singh, Basham, Nordeen, & Nordeen, 2000). Moreover, manipulations that extend sensitive periods can also delay developmental

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changes in NMDAR subunit expression and/or physiology (Carmignoto & Vicini, 1992; Chen, Cooper, & Mower, 2000; Mower, 1991; Philpot, Sekhar, Shouval, & Bear, 2001; Quinlan et al., 1999a, 1999b; Singh et al., 2000).

In zebra finches, vocal learning is restricted to a sensitive developmental period that parallels regionally specific changes in both the NR2B:NR2A ratio, and NMDAR function (Basham, Sohrabji, Singh, Nordeen, & Nordeen, 1999; Heinrich, Singh, Sohrabji, Nordeen, & Nordeen, 2002; Livingston & Mooney, 1997; Scott, Nordeen, & Nordeen, 2001; Singh et al., 2000; White, Livingston, & Mooney, 1999). During *sensory acquisition* (posthatch days (PHD) 25–60), young males memorize a song model. Then, during *sensory-motor learning* (PHD 35–120), birds practice song and gradually learn to mimic that model in their own vocalizations. Although the ratio of NR2B:NR2A mRNA decreases during both these phases of vocal learning in several brain regions implicated in song behavior, the steepest changes occur during acquisition (Heinrich et al., 2002; Scott et al., 2001; Singh et al., 2000).

With respect to regulating sensitive period duration, perhaps the most interesting changes in NMDAR gene expression occur within the lateral magnocellular nucleus of the anterior neostriatum (lMAN), a forebrain region implicated specifically in song development (Bottjer, Miesner, & Arnold, 1984; Scharff & Nottebohm, 1991; Sohrabji, Nordeen, & Nordeen, 1990). NMDAR activation in the lMAN is required for normal song learning (Basham et al., 1996) and during song acquisition NR2B mRNA in lMAN declines, while NR2A mRNA increases. In addition, early isolation from conspecific song delays the developmental decline in NR2B mRNA (without affecting NR1 or NR2A) specifically within the lMAN (Heinrich et al., 2002; Singh et al., 2000), while also delaying closure of the sensitive period (Aamodt, Nordeen, & Nordeen, 1995; Eales, 1987; Price, 1979). While the mechanism by which early song exposure affects NMDAR subunit expression is unknown, the lMAN is rich in androgen-accumulating neurons (Balthazart, Foidart, Wilson, & Ball, 1992; Nordeen, Nordeen, & Arnold, 1987), and isolation rearing transiently depresses androgen levels in young males (Livingston, White, & Mooney, 2000). Furthermore, premature androgen exposure actually accelerates developmental changes in lMAN NR2B (and NR2A) (Heinrich et al., 2002; Singh et al., 2000), and can impair song development (Korsia & Bottjer, 1991; Whaling, Nelson, & Marler, 1995).

These observations suggest that song and/or androgen-induced changes in lMAN NMDAR subunit expression may contribute to closing the sensitive period for acquisition by affecting the probability of developmentally regulated plasticity. Consistent with this view, there is a developmental reduction in the ability to induce NMDAR-dependent long-term potentiation within the lMAN (Boettiger & Doupe, 2001). There are at least two ways that changes in NMDAR composition could affect the threshold for this form of synaptic change. First, decreasing the NR2B:NR2A ratio reduces NMDAR current duration (Flint, Maisch, Weishaupt, Kriegstein, & Monyer, 1997; Kuehl-Kovarik, Magnusson, Premkumar, & Partin, 2000; Monyer et al., 1994; Tovar & Westbrook, 1999) thus affecting both synaptic integration times and postsynaptic Ca^{2+} influx (Hestrin, 1992; Hoffmann, Gremme, Hatt, & Gottmann, 2000). Indeed, within the lMAN such changes in NMDAR-mediated current kinetics are associated with the decline in the NR2B:NR2A ratio during the period of song learning (Livingston & Mooney, 1997; White et al., 1999). Moreover, this developmental decrease in NMDAR current duration is delayed by isolation rearing (see Fig. 1) and accelerated by early androgen exposure (Livingston et al., 2000; White et al., 1999; White & Mooney, 2000). However, recent studies strongly suggest that the developmental change in lMAN NMDAR current duration is not sufficient for

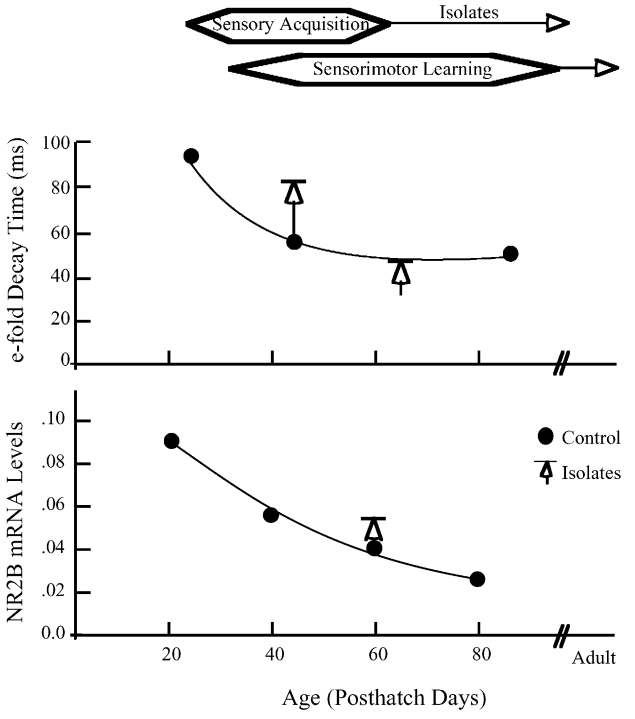

Fig. 1. A highly schematized depiction of the relationship between stages in song learning and the developmental decline in NMDAR-mediated current duration (upper panel) and NR2B hybridization levels (lower panel: *y*-axis units = grain density/somal area) in zebra finch lMAN. Early isolation from conspecific song extends the sensitive period for song learning (rightward arrows at top) and delays the developmental decline in NMDAR current duration and NR2B transcript levels. By PHD60-65, NMDA-EPSCs decay times are similar in isolates and controls, but NR2B mRNA levels in isolates remain elevated relative to controls. (Data are adapted from White et al., 1999, Livingston et al., 2000, and Singh et al., 2000.)

closing the sensitive period, as zebra finches can acquire song material weeks after NMDAR current kinetics have matured within the lMAN (Livingston et al., 2000; White & Mooney, 2000).

A second way that NMDAR composition could affect synaptic plasticity is by influencing interactions with intracellular proteins essential to synaptic change. For example, Ca^{2+} calmodulin-dependent protein kinase II (CaMKII), an enzyme essential to NMDAR-mediated long-term potentiation, preferentially associates with the NR2B subunit. Thus, decreases in this subunit could affect the efficiency of biochemical cascades that mediate changes in synaptic strength (Fukunaga, Muller, & Miyamoto, 1995; Fukunaga, Stoppini, Miyamoto, & Muller, 1993; Lisman, Malenka, Nicoll, & Malinow, 1997; Lisman & McIntyre, 2001; Nicoll & Malenka, 1999; Strack & Colbran, 1998) through mechanisms that are independent of changes in receptor current kinetics (e.g., see Barth & Malenka, 2001). This is an important concept because NR2B mRNA expression in lMAN neurons remains elevated beyond the time at which NMDAR current kinetics have matured (see Fig. 1) in both normal and isolation-reared birds (Livingston et al., 2000; Singh et al., 2000).

In the present study, we assessed whether prolonged elevation of lMAN NR2B is necessary for extending the sensitive period for song learning. We exposed isolation-reared birds briefly to either testosterone (T) or song, and found that these manipulations promote a normal developmental decline in lMAN NR2B mRNA. Yet, despite their "normal" pattern of NR2B expression, these birds exhibited an extended sensitive period for learning song. We conclude that the normal, experience-driven decrease in NR2B mRNA expression in lMAN is not sufficient to close this sensitive period.

2. Materials and methods

2.1. Subjects

2.1.1. Aviary reared controls

All control animals were raised on a 14:10 h light/dark schedule in free flight aviaries containing six breeding pairs and any offspring <PHD65. In our lab, these conditions typically foster the acquisition of songs heard between PHD25-60. All control animals remained in the aviaries until sacrificed for in situ hybridization or exposed to a tutor (see below).

2.1.2. Isolates

To extend temporally the capacity for new song learning, a separate set of birds was raised in isolation from conspecific song. At PHD9 isolates were removed from the aviary along with their clutchmates and parents. On PHD10 fathers were removed, and isolates were separated acoustically and visually from all adult males. At PHD30 isolates were moved to individual cages visually separated from each other until they were sacrificed.

2.1.3. T-isolates

A separate group of isolates was housed as described above and then treated with T to promote the downregulation of NR2B mRNA within the lMAN. At PHD24 T-treated isolates were implanted subcutaneously with a 10-mm Silastic tube filled with 7 mm of crystalline T (4-androsten-17β-ol-3-one; Steraloids, Newport, RI). At PHD30 the implant was removed, and animals were individually caged and visually isolated until they were sacrificed or exposed to a tutor.

2.1.4. Pre-exposed isolates

Pre-exposed isolates were raised in our free flight aviaries with access to male song until PHD30. At that time, juveniles were removed from the aviary and placed in individual cages, acoustically isolated from adult male song, and visually isolated from each other. They remained there until sacrificed or exposed to a tutor.

All groups consisted of 5–7 birds.

2.2. In situ hybridization

Isotopic in situ hybridization for NR2B mRNA was carried out as previously described (see Singh et al., 2000). Coronal sections thru the lMAN were hybridized with a 45-base oligonucleotide probe, processed for autoradiographic detection of hybridization, and then lightly stained with thionin. To control for variability in probe labeling and autoradiography between multiple hybridization experiments, all analyses and comparisons were restricted to tissue run within a single experiment. Birds sacrificed at PHD40 (isolates, T-isolates, pre-exposed isolates, and controls) were run together with PHD45 isolates and controls. Birds sacrificed at PHD65 (T-isolates, pre-exposed isolates, and controls) were processed in a separate experiment. The regional specificity, and overall hybridization levels were visually similar to those described in previous developmental studies of NR2B mRNA in zebra finch (Basham et al., 1999; Singh et al., 2000).

2.2.1. Analysis

Procedures for analysis of mRNA levels in zebra finch tissue have been described previously (Heinrich et al., 2002; Singh et al., 2000). Briefly, using a computer-assisted image analysis system (Image, NIH), we calculated "silver grain area/total somal area" from two randomly selected sections containing the lMAN. In each section, two adjacent fields were sampled from the approximate center of the nucleus in each hemisphere, yielding eight measurements for each animal. Within

each sample field, total somal area and somal area occupied by silver grains were measured at 40× magnification. The silver grain area measurement was based on gray-level thresholding after placing a blue filter (transmission wavelengths $\leqslant$490 nm) over the light source to render the Nissl staining invisible. Background grain density was determined by calculating the average of eight measurements (area occupied by silver grains/total area) taken from nontissue portions of the slides, and was then subtracted from the somal grain density for each animal. For some groups, lateral neostriatum (a non song region ventrolateral to lMAN and lateral to Area X) was measured similarly.

The effects of isolation at PHD40 and 45 (run together in one hybridization experiment) on lMAN somal grain density were evaluated by a two way ANOVA (age × rearing condition). The effects of rearing/hormones at PHD40 and 65 were evaluated by independent *t* tests (2-tailed) on selected groups (Bonferroni corrected).

2.3. Behavior

For behavioral analysis six additional animals in each of the control, T-isolate and pre-exposed isolate groups were raised as described above and then individually exposed to a tutor between PHD65 and 90. Each tutor was utilized in all three groups, and tutors were assigned so as to maximize the dissimilarity between pupil and tutor song at the onset of tutoring. During tutoring, each juvenile was housed with the tutor and was visually isolated from other birds except for a stimulus female housed in an adjacent cage. At PHD90 the pupils were placed individually in a cage visually isolated from other males, and acoustically isolated from their tutor.

Female-directed song was recorded at PHD65, 90, and 120 at a sampling rate of 22,050 Hz using Avisoft Recorder software. Analyses of song spectrograms were carried out by two independent observers, each blind to the experimental conditions. A visual method of comparison that is used routinely in our laboratory (Aamodt, Nordeen, & Nordeen, 1996; Basham et al., 1996; Sohrabji et al., 1990) was used to assess song similarity. Song bouts from each bird recorded at PHD120 were compared to tutor's song. Individual syllables were defined as acoustic units ($\geqslant$20 ms) surrounded by intervals of baseline energy lasting at least 10 ms except in cases of abrupt frequency transitions (>1 kHz) where the intersyllable interval could be as short as 5 ms. Each syllable from the pupil's song was matched to the syllable in the tutor's song that it most closely resembled. The phonological similarity of the pair then was scored on a 0–3 scale (0 = no similarity, 1 = slight similarity, 2 = highly similar, 3 = matched). A score of 0 was assigned when the tutor's song did not contain a syllable of the same type (downsweep, stack, click, high note, other: defined by Williams & Staples, 1992). A score of 1 was assigned to syllable pairs that exhibited either a major discrepancy in fundamental frequency, contour, or duration or minor differences in two or more of these acoustic parameters. A score of 2 was assigned when there was a minor difference in only one of these acoustic parameters, and a score of 3 was reserved for syllable pairs that were virtually identical in all respects. An average syllable score then was calculated for each animal. Two-tailed Mann–Whitney *U* tests were used to evaluate differences between average syllable scores for each group.

Learned/copied syllables were then operationally defined as syllables that received a score of 2 or 3 (there was 89% agreement between scorers on whether or not a syllable was scored as learned). Scores of syllable similarity were separated into two different measures of "song learning." Percentage of song inspired by tutor was defined as the number of pupil syllables scored as learned divided by the total number of pupil syllables. The percent of tutor's song imitated was defined as the number of tutor syllables copied divided by the total number of tutor syllables available to the pupil. For both measures of song learning, two-tailed Mann–Whitney *U* tests were used to evaluate differences between group means.

3. Results

3.1. Effects of isolation on NR2B mRNA in lMAN at PHD40 and 45

A two way (age × treatment) randomized ANOVA revealed a significant main effect of treatment on NR2B mRNA expression at PHD40 and 45 [$F(1,16) = 10.19$, $p < .01$]. There was no main effect of age and no interaction. Overall, isolates at PHD40 and 45 exhibited NR2B mRNA levels that were 17% higher than in age-matched controls (Fig. 2: data presented as percent of control). The early effects of isolation appeared to be regionally specific, as levels of NR2B mRNA in the lateral neostriatum of isolates were not significantly different than controls (data not shown). These findings extend the previous report that at PHD60, NR2B mRNA expression in zebra finch lMAN is higher in isolation-reared birds than in controls (data reproduced in right side of Fig. 2).

3.2. NR2B levels are normalized by T-treatment of isolates or limited song exposure

Normally reared birds that are treated with exogenous T experience an accelerated downregulation of NR2B mRNA and often exhibit abnormalities in song development (Korsia & Bottjer, 1991; Singh et al.,

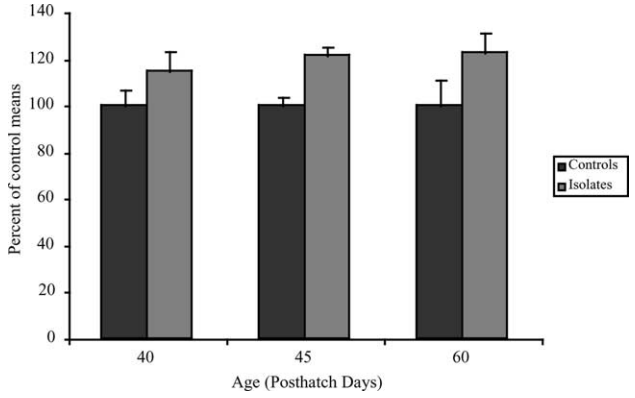

Fig. 2. Early isolation (beginning on PHD9) from conspecific song alters NR2B mRNA expression in lMAN. An overall ANOVA on PHD40 and 45 timepoints reveals that transcript levels are significantly higher in isolates than in controls at PHD40 and 45 [$F(1,16) = 10.19$, $p < .01$] ($n = 5$ per group). For illustration purposes data are presented here as percent of control values ± SEM. For comparison, the effect of isolation at PHD60 (published previously (Singh et al., 2000)) is also shown.

2000). Here, we report that brief (6 days) T-treatment also can counteract the effects of isolation on lMAN NR2B levels. That is, while isolation normally delays the downregulation of NR2B expression within lMAN, isolate birds that are treated with T exhibit levels of NR2B expression that are at control values by PHD40 (Fig. 3, left).

Although song exposure only until PHD25 delays the physiological maturation of the NMDAR (Livingston et al., 2000), and exposure until PHD35 can be inadequate for normal song development (Eales, 1985), we found that access to male song until PHD30 is sufficient to protect against the effects of subsequent isolation on NR2B mRNA expression in lMAN. At PHD40, pre-exposed isolates had NR2B mRNA levels in lMAN that were comparable to controls (Fig. 3, left).

The normalizing effects of T-treatment or early limited song exposure on NR2B expression persist through PHD65 (Fig. 3, right). In fact, student t tests (Bonferroni adjusted) revealed that at this age, NR2B message levels in lMAN of T-treated isolates were below control values ($p < .01$), as were levels in pre-exposed isolates ($p < .05$). These treatment effects were regionally specific: NR2B transcript levels within the lateral neostriatum of T-isolates and pre-exposed isolates were not significantly different from controls.

3.3. Song acquisition despite NR2B mRNA at or below control levels

Although both the T-isolation and limited song exposure manipulations successfully counteracted the effects of isolation on NR2B mRNA in lMAN, animals in these conditions remained capable of song learning at a time when normally reared animals were not (Fig. 4). Groups did not differ significantly in the average number of syllables produced at PHD120, (controls = 5.7 ± .23, pre-exposed isolates = 6.5 ± .62, T-isolates = 5.5 ± .68). However, T-isolates imitated a larger proportion of the tutor's song ($z = 2.88$, $p < .01$), and derived more of their song from the tutor ($z = 3.04$, $p < .01$) than did controls. Likewise, pre-exposed isolates imitated a larger proportion of the tutor's song ($z = 2.48$, $p < .015$) and more of their syllables were accurately copied from the tutor ($z = 2.72$, $p < .01$), as compared to controls birds. Whereas 4 out of 6 T-treated isolates and 5 out of 6 pre-exposed isolates produced 2 or more "learned" syllables, only 1 of the 6 control birds reproduced more than 1 tutor syllable. The overall similarity scores for T-isolates (1.3 + .16), and

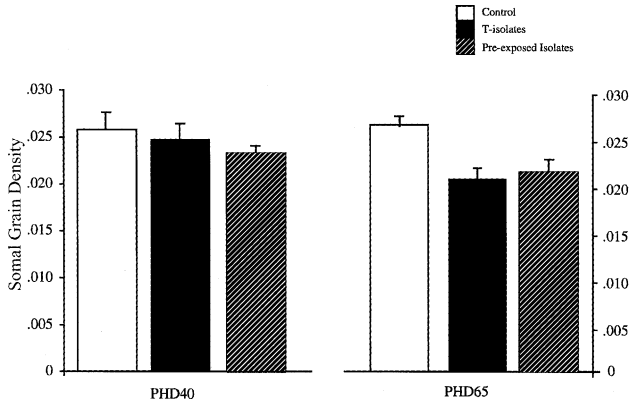

Fig. 3. Early testosterone treatment (PHD24–30) or limited song exposure (until PHD30) normalize NR2B mRNA levels in lMAN at both PHD40 (left) and at PHD65 (right). At PHD 40, both the T-isolates and pre-exposed isolates expressed levels of NR2B mRNA in lMAN that were similar to controls. At PHD65, NR2B levels in both T-isolates and pre-exposed isolates were significantly lower than controls ($p < .01$ and $p < .05$, respectively). Data shown are means ± SEM. PHD40 and PHD65 data are from separate hybridization experiments.

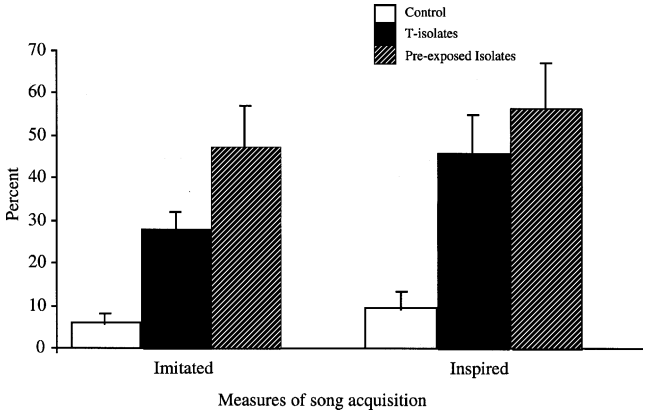

Fig. 4. When tutored between PHD65 and 90, both T-isolates and pre-exposed isolates imitated a greater proportion of the tutor's song (left) and produced a greater percentage of song inspired by the tutor (right) than did controls. Data shown are group means ± SEM.

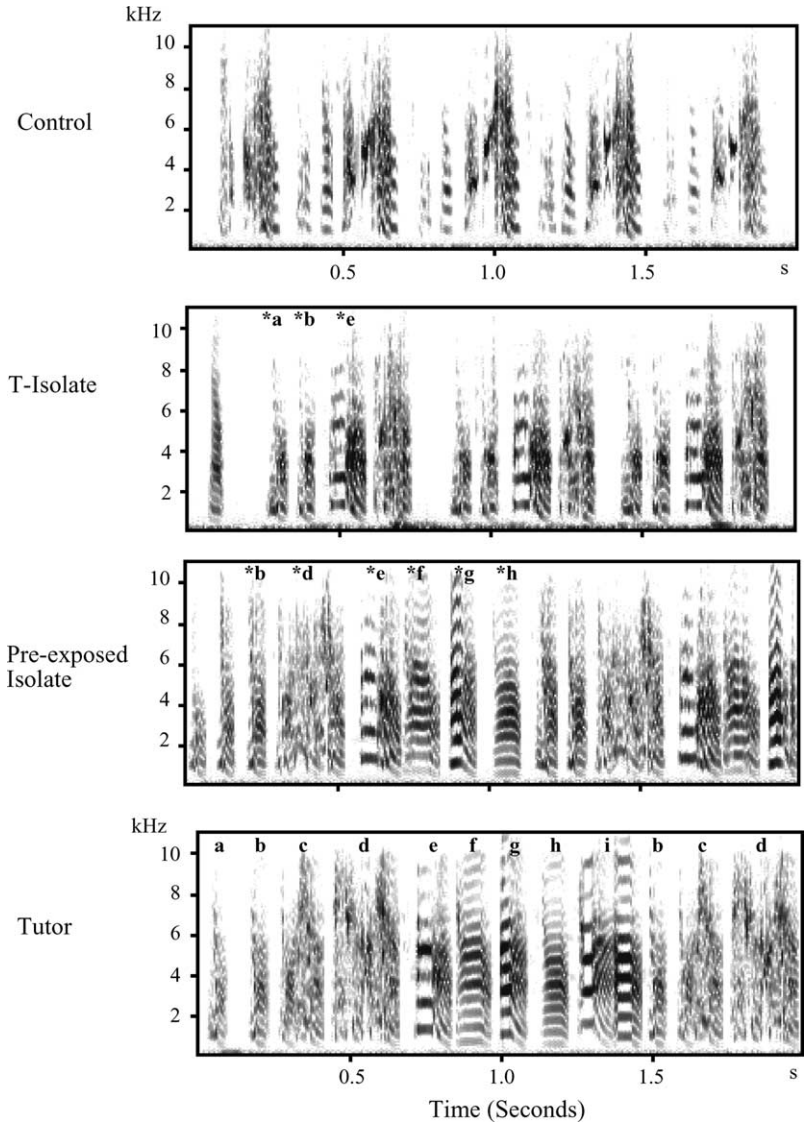

Fig. 5. Sonograms illustrating imitation of a tutor heard between PHD65 and 90. Songs produced at PHD120 are shown for a representative control (top), T-isolate (2nd), and pre-exposed bird (3rd) along with the tutor's song (bottom). Each of the tutor's syllables are denoted with a letter below the bottom sonogram. Syllables scored as learned by the pupils are indicated by an asterisk (*), and lettered to correspond to the tutor's syllable that they most closely resembled.

pre-exposed isolates ($1.8 + .18$) also were significantly higher than for controls ($0.8 + .17$; $z = 2.00$, $p < .05$, $z = 2.88$, $p < .01$, respectively). Example sonograms following tutor exposure in a T-isolate, pre-exposed isolate, and control zebra finch are shown in Fig. 5.

4. Discussion

In earlier work, we demonstrated that lMAN neurons exhibit a developmental decrease in NR2B expression that roughly parallels the sensitive period for song learning. Moreover, isolation from conspecific song produces both extends the sensitive period for vocal learning, and delays the developmental decline in lMAN NR2B mRNA expression (see Fig. 1). These observations suggested that vocal learning might require a threshold level of NR2B expression within this forebrain song region, and that the normal developmental decrease in NR2B expression contributes to closing the sensitive period for learning. A similar view has been advanced for other instances of developmentally regulated plasticity (Carmignoto & Vicini, 1992; Nase, Weishaupt, Stern, Singer, & Monyer, 1999; Ramoa & Prusky, 1997). However, the present results do not support this view. In two separate groups of birds deprived of normal song exposure, the sensitive period for learning was extended despite normal (or supernormal) declines in lMAN NR2B expression. These data complement previous work of White and Mooney (2000)

indicating that song acquisition can occur well after maturation of NMDAR EPSCs duration, and suggest that neither experience-dependent changes in NMDAR physiology nor subunit composition within the developing lMAN are sufficient to close the sensitive period for vocal learning.

Developmental changes in NMDAR subunit expression are evident throughout the song system, but in the present study we have focused attention on lMAN because: (1) NMDAR expression here, but not elsewhere in the song system, is known to be affected by manipulations that extend the sensitive period, and (2) NMDAR activation in this region is critical to normal song learning (Basham et al., 1996). In demonstrating that the developmental reduction in lMAN NR2B expression does not preclude subsequent vocal learning two questions emerge: (1) what role, if any, does this experience-dependent developmental change in NMDAR gene expression play in song learning and (2) what other neural changes (in the lMAN or elsewhere) might be responsible for curtailing the period of vocal learning.

Speculation that NMDAR maturation may limit developmental plasticity has come in part from the fact that a developmental decrease in NR2B levels and an accelerated decay of the NMDAR EPSC would both tend to increase the threshold for Hebbian based synaptic strengthening (e.g., by reducing synaptic coincidence and the temporal integration of calcium influx). Consistent with this notion, Tang et al. (1999) demonstrated that adult mice overexpressing NR2B exhibit a reduced threshold for long term potentiation induction at hippocampal synapses. But in a developing neural network where information may be represented in the specific pattern of connections that is retained from an initially exuberant set, an increase in the threshold for LTP (thus favoring LTD, and synaptic elimination) may aid in learning. Thus, increased attention is being directed towards the hypothesis that changes in NMDAR function may actually contribute to opening, rather than curtailing, sensitive periods for plastic change. That is, an experience-driven refinement of immature circuitry may be enabled by the more stringent threshold for synaptic strengthening that results from faster current kinetics and/or less permissive conditions for intracellular signaling cascades. Consistent with this view, throughout the developing song system (and in mammalian visual cortex) the steepest changes in NMDAR subunit composition and physiology occur towards the opening of the critical period (Cao et al., 2000; Heinrich et al., 2002; Livingston et al., 2000; Roberts & Ramoa, 1999; Singh et al., 2000). Furthermore, in visual cortex these changes in the NMDAR are triggered rapidly by initial exposure to light, and clearly do not compromise subsequent plasticity (Quinlan et al., 1999a, 1999b).

These ideas predict that learning would be compromised or delayed if experience-dependent changes in lMAN NMDAR composition are blocked. Indeed, studies of mammalian visual cortex indicate that manipulations that delay NMDAR maturation (i.e., dark rearing) do delay the onset, as well as closure of the critical period for ocular dominance plasticity (Mower, 1991). An effective means of testing the functional significance of changes in NMDAR composition and function within the song system may be to block androgen action within the lMAN. That is, song exposure may regulate lMAN NMDAR subunit expression via an androgen-regulated process, since early isolation from song attenuates androgen levels in zebra finches (Livingston et al., 2000), and brief androgen exposure appears to be an effective substitute for song exposure in triggering changes in lMAN NMDAR subunit expression (but see White et al., 1999). Androgen-accumulating cells are abundant in the lMAN of zebra finches even early in song development (Nordeen et al., 1987), and in birds that display seasonal vocal plasticity in adulthood, changes in lMAN NR2B mRNA accompany changes in androgen levels (Singh et al., 2001).

Before considering other developmental changes that may be responsible for curtailing vocal learning, it is important to stress that current evidence has not established that sensitive period closure is *independent* of developmental changes in lMAN NMDAR composition and/or physiology, only that these receptor changes are not sufficient for terminating this learning period. Recent work in the mammalian somatosensory cortex, however, does suggest that the developmental regulation of plasticity is independent of some changes in the NMDAR. In NR2A −/− mice, Lu et al. (2001) have shown that the critical period for LTP induction and plasticity in barrel cortex closes at the normal time, despite the fact that NMDAR current kinetics remain slow and the normal developmental increase in the NR2A:NR2B ratio (defined pharmacologically) does not occur. Thus, the thalamocortical sensitive period can close independently of changes in the NR2A subunit and NMDAR current kinetics, although a role for developmental changes in NR2B has not been ruled out.

Notwithstanding the current findings, developmental changes in NMDAR-mediated synaptic plasticity within the lMAN remain worthy candidates for regulating the sensitive period. Several developmentally regulated synaptic events within the lMAN require NMDAR activation. The propensity for NMDAR-mediated synaptic potentiation among the synapses of recurrent lMAN axon collaterals decreases markedly between 20 and 60 days of age (Boettiger & Doupe, 2001). Furthermore, recent electrophysiological evidence is consistent with a decrease in the incidence of so-called "silent synapses" with the lMAN over the song learning period (Grammer & Bottjer, 2001). In other developing systems the loss of

silent synapses is attributed to the incorporation of AMPAR receptors into synapses that previously contained only NMDARs (Isaac, Crair, Nicoll, & Malenka, 1997). Interestingly, a similar recruitment of AMPA receptors has been suggested to underlie the maintenance of NMDAR-mediated LTP (Feldman, Nicoll, & Malenka, 1999; Rumpel Hatt & Guttmann). It will be important to learn if early isolation from song delays either the loss of NMDAR-mediated LTP or AMPAR recruitment within the lMAN. If so, then closure of the sensitive period may relate to alterations in biochemical cascades downstream of NMDAR activation. For example, as demonstrated in other systems, changes in the efficacy of CaMKII, PKA (reviewed in Kind & Neumann, 2001), CREB (Mower, Liao, Nestler, Neve, & Ramoa, 2002; Sakaguchi, Wada, Maekawa, Watsuji, & Hagiwara, 1999), or calcineurin (Krupp, Vissel, Heinemann, & Westbrook, 1996; Shi, Townsend, & Constantine-Paton, 2000) within lMAN neurons could restrict plasticity, as could changes in the expression of genes associated with synaptic plasticity (e.g., CPG-15 (Lee & Nedivi, 2002), calbindin (Braun, Scheich, Heizmann, & Hunziker, 1991; Wild, Williams, & Suthers, 2001), or BDNF (Huang et al., 1999; Lin et al., 1998)).

Additionally, it will be important to look to other song-related areas for developmental changes that could temporally restrict vocal learning. There is little doubt that song acquisition engages a number of brain regions, and striking developmental changes in gene expression occur in many song nuclei, as do song-induced changes in gene expression (Jarvis, Schwabl, Ribeiro, & Mello, 1997; Mello & Clayton, 1994; Mello, Vicario, & Clayton, 1992; Nastiuk, Mello, George, & Clayton, 1994). Emerging technologies that allow for manipulating these changes will be essential to understanding how they contribute to the developmental regulation of vocal learning.

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